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3. Jarvis et al
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4. Jarvis et al
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5. Kenny et al
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Proc. Natl. Acad. Sci. USA
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Microbiology

Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation

(bacterial pathogenesis/protein secretion/signal transduction)

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ABSTRACT **Enteropathogenic *Escherichia coli* (EPEC) causes a characteristic histopathology in intestinal epithelial cells called the attaching and effacing lesion. Although the histopathological lesion is well described the bacterial factors responsible for it are poorly characterized. We have identified four EPEC chromosomal genes whose predicted protein sequences are similar to components of a recently described secretory pathway (type III) responsible for exporting proteins lacking a typical signal sequence. We have designated the genes *sepA*, *sepB*, *sepC*, and *sepD* (*sep*, for secretion of *E. coli* proteins). The predicted Sep polypeptides are similar to the Lcr (low calcium response) and Ysc (*yersinia* secretion) proteins of *Yersinia* species and the Mxi (membrane expression of invasion plasmid antigens) and Spa (surface presentation of antigens) regions of *Shigella flexneri*. Culture supernatants of EPEC strain E2348/69 contain several polypeptides ranging in size from 110 kDa to 19 kDa. Proteins of comparable size were recognized by human convalescent serum from a volunteer experimentally infected with strain E2348/69. A *sepB* mutant of EPEC secreted only the 110-kDa polypeptide and was defective in the formation of attaching and effacing lesions and protein-tyrosine phosphorylation in tissue culture cells. These phenotypes were restored upon complementation with a plasmid carrying an intact *sepB* gene. These data suggest that the EPEC Sep proteins are components of a type III secretory apparatus necessary for the export of virulence determinants.**

Enteropathogenic *Escherichia coli* (EPEC) causes infantile diarrhea throughout the world. EPEC infections result in the formation of attaching and effacing (AE) lesions which are characterized by effacement of intestinal microvilli, intimate adherence of bacteria to enterocytes, and accumulation of polymerized actin and other cytoskeletal components in the eukaryotic cell. Filamentous actin accumulates below the bacteria, resulting in the formation of cup-like pedestals (1, 2). Several signal transduction mechanisms have been associated with AE lesion formation, including tyrosine phosphorylation of a 90-kDa host cell protein (Hp90) (3), fluxes in inositol phosphate levels (4), increased intracellular Ca^{2+} levels (5), and phosphorylation of myosin light chain (6). We recently described a large (35-kb) region in the EPEC chromosome, termed LEE (locus of enterocyte effacement), that encodes all of the virulence determinants for AE lesion formation so far identified (7). Two chromosomal loci within the LEE, *eaeA* and *eaeB* (*eae*, for *E. coli* attaching and effacing), have been characterized (8, 9). The *eaeA* gene encodes a 94-kDa outer membrane protein, intimin, which has sequence homology to the invasin protein of *Yersinia* species (8). An *eaeA* mutant of EPEC is unable to form AE lesions in cultured cells (8, 10) but can still induce host cell tyrosine phosphorylation of Hp90 and fluxes in inositol phosphate levels (3, 4). When tested in an experimental human model of EPEC infection, the *eaeA* mutant showed reduced virulence (11). The *eaeB* gene, located 4.4 kb downstream of *eaeA* on the EPEC chromosome (9), encodes a factor involved in signal transduction in epithelial cells (12). An *eaeB* deletion mutant of EPEC is unable to induce AE lesion formation in eukaryotic cells but is still capable of producing intimin (9). This mutant is also incapable of inducing Hp90 phosphorylation and the release of inositol phosphates from HeLa cells (12).

Two Tn*phoA* mutants of EPEC strain E2348/69 [27-3-2(1) and 14-2-1(1)] have phenotypes similar to the *eaeB* deletion mutant. These mutants (called *cfm*, for class four mutants) are noninvasive and are unable to induce Hp90 phosphorylation or trigger inositol phosphate fluxes (3, 4, 13). Here we identify one of the interrupted genes in *cfm* 27-3-2(1) and describe three additional open reading frames in the LEE region of the EPEC chromosome.** We hypothesize that these genes are involved in the secretion of virulence factors in EPEC via a newly described type III secretory apparatus.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* E2348/69 is a prototypic EPEC strain which has been extensively characterized in volunteer studies (14, 15). Strain 27-3-2(1) is a *cfm* mutant of E2348/69 containing two chromosomal Tn*phoA* insertions (13). *E. coli* HS-4 is a nonpathogenic fecal isolate previously shown to be avirulent in volunteers (16).

DNA Sequencing. Cloned DNA fragments in plasmids pCVD446, pCVD459, and p27W [a Tn*phoA* fusion junction subclone from 27-3-2(1)] (Fig. 1) were sequenced by use of the Ready Reactions DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) and an Applied Biosystems model 373A automated sequencer. Nested deletions of pCVD447B (Fig. 1) were sequenced by the Sanger dideoxy method (Sequenase version 2.0 kit; United States Biochemical). DNA sequence

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Abbreviations: EPEC, enteropathogenic *Escherichia coli*; AE, attaching and effacing; LEE, locus of enterocyte effacement; FAS, fluorescence actin staining; LA, localized adherence.

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**The sequences reported in this paper have been deposited in the GenBank database (accession nos. Z49933 and Z49972).

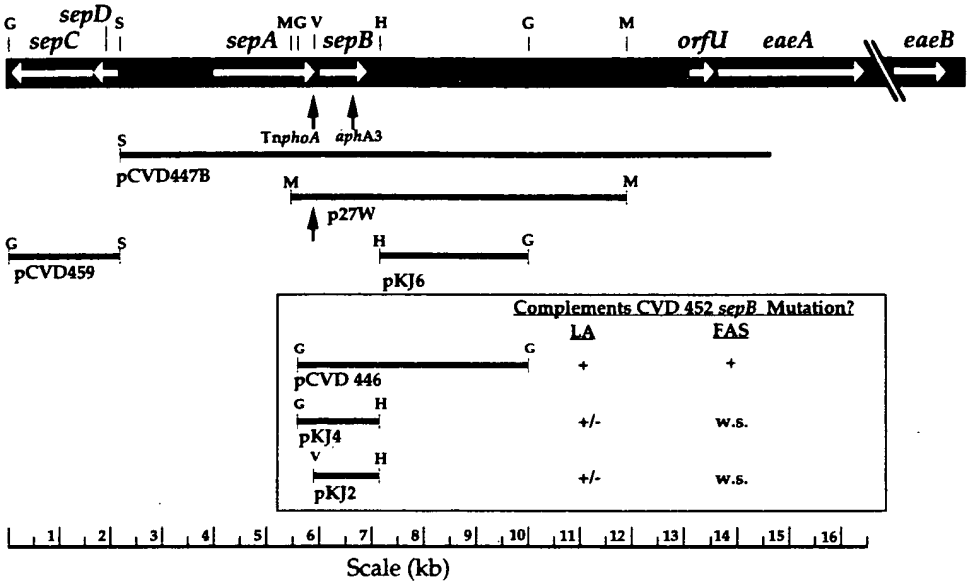

FIG. 1. Map showing the *sep* and *eae* genes in the EPEC LEE region. Subclones used to generate DNA sequence include: pCVD447B, a 12.5-kb *Sal* I subclone from pJY3 (8) containing 4.6 kb of Tn*phoA* (not shown) from an insertion in *eaeA*; pCVD459, a 2.2-kb *Bgl* II–*Sal* I subclone from p30 (13); p27W, a 14-kb *Mlu* I subclone containing one of the Tn*phoA* insertions (indicated by arrow) from *cfm* 27-3-2(1) (13) (actual size is 7.6 kb greater than shown due to presence of Tn*phoA*); and pCVD446, a 4.5-kb *Bgl* II subclone from pCVD436 (8). The *aphA3* insertion in the *sepB* gene is indicated (arrow). The box shows the localized adherence (LA) and fluorescence actin staining (FAS) complementation data for pCVD446, pKJ2 (1.2-kb *Eco*RV–*Hin*dIII), and pKJ4 (1.5-kb *Bgl* II–*Hin*dIII); w.s., weak shadow. Relevant restriction enzyme sites: G, *Bgl* II; H, *Hin*dIII; M, *Mlu* I; S, *Sal* I; V, *Eco*RV. The hatching between *eaeA* and *eaeB* represents 4.4 kb. Open arrows show direction of transcription.

analysis was performed with programs from the Genetics Computer Group (University of Wisconsin, 1991).

Mutagenesis and Genetic Techniques. An 850-bp cassette carrying a kanamycin-resistance gene (*aphA3*) (17) was used to create an insertion mutation in the *sepB* gene of E2348/69. This cassette contains a ribosomal binding site and an ATG start site at the 3′ end so that translation of sequences downstream of the insertion is maintained. Plasmid pKJ2, a 1261-bp *Eco*RV–*Hin*dIII fragment from pCVD446 (Fig. 1), was digested with *Eco*RI and the *aphA3* cassette was inserted. The mutated *sepB* gene was introduced into a streptomycin-resistant derivative of E2348/69 by allelic exchange using the suicide vector pCVD442 (10) to create CVD452.

Protein Analysis. Culture supernatants were analyzed as follows: strains were grown in Eagle's minimal essential medium at 37°C shaking to an OD_{600} of 1.0. Bacteria were pelleted by centrifugation (10,000 × *g*, 10 min), and phenylmethylsulfonyl fluoride (50 μg/ml; Sigma), aprotinin (0.5 μg/ml; Sigma), and EDTA (0.5 μM; Sigma) were added to the supernatants, which were then passed through a 0.45-μm filter and precipitated overnight by adjusting the solution to 100% saturation with ammonium sulfate at 4°C. The precipitated proteins were pelleted by centrifugation (27,500 × *g*, 5 hr, 4°C) and dialyzed against phosphate-buffered saline (PBS). After 10-fold concentration with polyethylene glycol the proteins were analyzed by SDS/PAGE.

Tissue Culture Assays. Assays for HEp-2 LA (18), FAS (1), and induction of protein tyrosine phosphorylation (3) were performed as described.

Rabbit Immunizations. Polyclonal antiserum was raised against the proteins secreted from EPEC. Four polypeptides (38, 28, 25, and 23 kDa) were excised from SDS/polyacrylamide gels, and the gel slices were lyophilized, macerated, and suspended in PBS. A New Zealand White rabbit was immunized subcutaneously with the antigen mixture in an equal volume of complete Freund's adjuvant. Two booster injections in incomplete Freund's adjuvant were given. The second booster contained 50 μg of total EPEC secreted proteins.

Immunoblotting. Proteins were separated by SDS/PAGE and electrophoretically transferred to Immobilon-P poly(vinylidene difluoride) membranes (Millipore). Immunoblots were probed with monoclonal anti-phosphotyrosine 4G10 (Upstate Biotechnology, Lake Placid, NY) (1:2000), rabbit serum raised against EPEC secreted proteins (1:1000), or human volunteer sera (15) drawn prior or 28 days after experimental infection with EPEC E2348/69 (1:200). Bound antibody was detected with horseradish peroxidase-conjugated secondary antibodies including anti-rabbit IgG, anti-mouse IgG (Sigma), and anti-human IgG (Kirkegaard & Perry Laboratories), followed by enhanced chemiluminescence substrate (ECL; Amersham) and autoradiography.

RESULTS

Sequence Analysis. McDaniel *et al.* (7) determined that one of the two Tn*phoA* insertions in *cfm* 27-3-2(1) is within a 35-kb region of the EPEC chromosome designated LEE. A 12-kb region of LEE upstream of *eaeA* was sequenced by use of plasmids pCVD447B, p27W, pCVD459, and pCVD446 (Fig. 1). The predicted polypeptides of four open reading frames within LEE showed similarities to components of type III secretion pathways involved in the export of bacterial virulence factors. These open reading frames were designated *sepA*–*sepD* (Fig. 1). The relevant features of the *sep* genes and their putative products are described below and summarized in Table 1.

The *sepA* gene, located 9.7 kb upstream of *eaeA* (Fig. 1), encodes a predicted 72-kDa polypeptide. SepA is similar to the LcrD family of proteins in *Yersinia* (19), *Shigella* (20), and *Salmonella* (21), which are necessary for bacterial protein export and invasion into epithelial cells (Table 1). The hydropathy plots of SepA and predicted homologs show them to be inner membrane proteins with six to eight membrane-spanning domains (data not shown) (19–21). One of the Tn*phoA* insertions in cfm 27-3-2(1) was localized to *sepA* by sequencing the Tn*phoA*–EPEC junction in p27W by use of a primer specific for Tn*phoA* (Figs. 1 and 2).

Table 1. Type III secretory proteins in EPEC and other human pathogens

EPEC protein	Homologous proteins*			Putative location	Motif†
	Yersinia	*Shigella*	*Salmonella*		
SepA	LcrD [46, 72]	MxiA [43, 69]	InvA [40, 65]	Inner membrane	
SepB	YscN [44, 66]	Spa47 [35, 59]	SpaL (InvC) [48, 69]	—	ATP/GTP-binding site
SepC	YscC [31, 54]	MxiD [33, 51]	InvG [29, 52]	Outer membrane	Type II secretion, ATP/GTP-binding site
SepD	YscJ [36, 56]	MxiJ [27, 48]	—	Outer membrane	Lipoprotein signal peptidase II cleavage site

*Percent identity and similarity to Sep proteins determined with the GAP program from the Genetics Computer Group is given in brackets.
†Determined with the MOTIFS program from the Genetics Computer Group.

The *sepB* gene is located 84 bp downstream from *sepA* (Fig. 1) and encodes a predicted 31.5-kDa protein with similarity to proteins in *Yersinia* (22), *Shigella* (23), and *Salmonella* (24, 25) which are hypothesized to supply ATP for protein secretion (Table 1). These polypeptides contain Walker-box consensus sequences, which are found in ATP- and GTP-binding proteins (26). The Walker-box consensus sequences present in SepB (Fig. 2) and the homologs in Table 1 are similar to motifs found in the catalytic $F_1\beta$ subunit of the F_0F_1 ATP synthases in mitochondria, chloroplasts, and *E. coli*, which bind ADP and P_i and catalyze ATP synthesis via a proton-motive force (26, 27).

The *sepC* gene, which encodes a predicted 56-kDa polypeptide, is located 2.3 kb upstream of *sepA* and is transcribed in the opposite orientation from *sepA* and *sepB* (Fig. 1). The predicted SepC polypeptide is similar to outer membrane proteins necessary for protein export in *Yersinia* (28) and *Shigella* (29) and for epithelial cell invasion by *Salmonella* (30) (Table 1). The carboxyl-terminal region of the predicted SepC polypeptide has an ATP/GTP-binding-site motif A (Walker box A) contained within a bacterial type II secretion system consensus sequence (Table 1).

The *sepD* gene is located 1.0 kb upstream of *sepC* and, like *sepC*, is transcribed in the opposite direction from *sepA* and *sepB* (Fig. 1). The predicted 21-kDa polypeptide encoded by *sepD* contains a signal peptidase II cleavage motif common to lipoproteins (Table 1). The putative SepD polypeptide is similar to outer membrane lipoproteins of *Shigella* (31) and *Yersinia* (28) (Table 1).

Secreted Proteins of EPEC E2348/69 and CVD452. Culture supernatants of EPEC strain E2348/69 contain a polypeptide with an apparent molecular mass of 110 kDa and several smaller polypeptides ranging from 38 to 19 kDa (Fig. 3*A*, lane 2). A *sepB* mutant of E2348/69 was constructed and designated CVD452. Analysis of culture supernatants of CVD452 and E2348/69 shows that the *sepB* mutation eliminates the secretion of all but the 110-kDa polypeptide (Fig. 3*A*, lane 3). The polypeptides between 94 and 67 kDa in E2348/69 and CVD452 are also present in culture supernatants of the nonpathogenic *E. coli* strain HS-4 and may be due to autolysis (Fig. 3*A*, lane 1).

Immunoblots with Rabbit Serum. Immunoblot analysis using rabbit antiserum raised against a mixture of the proteins secreted by E2348/69 confirmed that the 38- to 19-kDa proteins were not secreted by CVD452 (Fig. 3*B*, lane 3). Plasmid pCVD446 (Fig. 1), containing the cloned wild-type *sepB* gene, restored protein secretion in CVD452 (Fig. 3*B*, lane 4). Three smaller *sepB* subclones—pKJ2, pKJ4, and pKJ6 (Fig. 1)—were unable to restore protein secretion in CVD452 (Fig. 3*B*, lanes 5–7). The antiserum detected two secreted proteins, 110 and 67 kDa, that were present in the culture supernatants of both E2348/69 and the *sepB* mutant CVD452 (Fig. 3*B*). The 110-kDa protein was not present in the normal flora strain HS-4. The 67-kDa protein was detected in HS-4 (Fig. 3*B*, lane 1) and in DMEM (data not shown). Western blot analysis of whole cell extracts of E2348/69 and CVD452 showed that antiserum prepared against the secreted proteins recognized similar proteins in both strains (Fig. 3*C*). An interesting exception, however, was a 38-kDa protein present in E2348/69 but absent from the *sepB* mutant. This result suggests that an intact type III secretion system may be necessary for the production or stability of this protein. Another interesting observation is that although the antiserum recognizes the

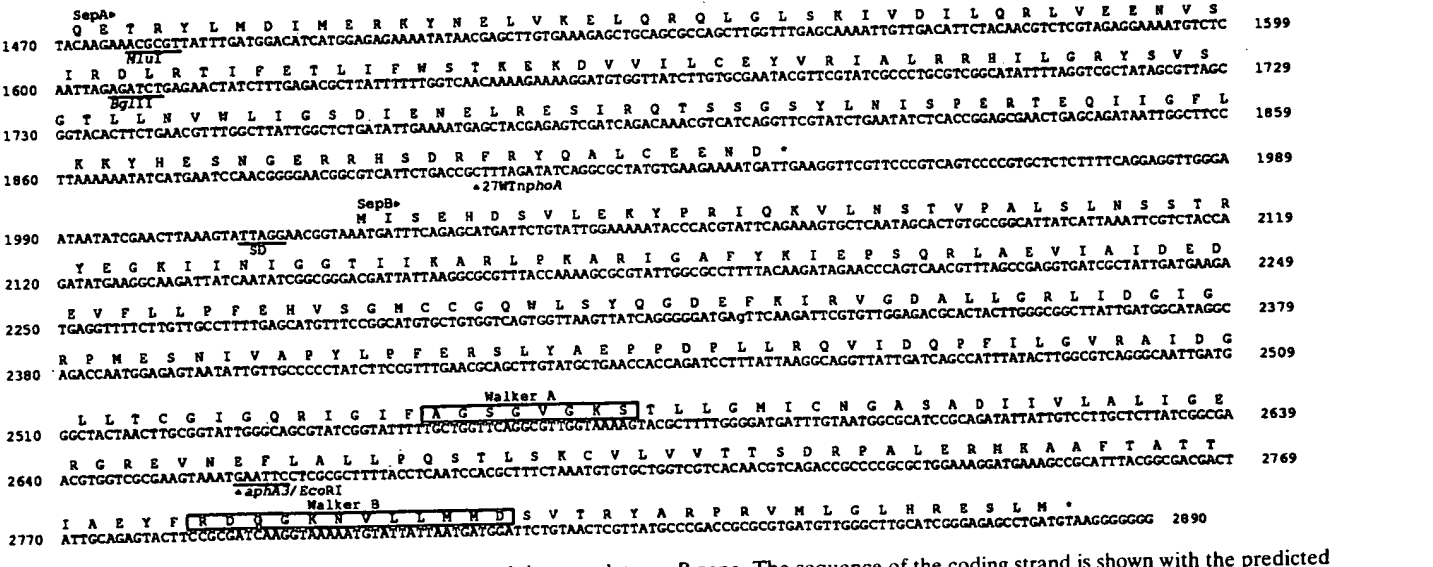

FIG. 2. Nucleotide sequence of the 3′ end of *sepA* and the complete *sepB* gene. The sequence of the coding strand is shown with the predicted protein sequence. Relevant restriction sites are underlined. The Tn*phoA* insertion site in *sepA* and the *aphA3* insertion site in *sepB* are indicated. A putative ribosomal binding site (Shine–Dalgarno, SD) sequence upstream of *sepB* is underlined and the Walker box A and B consensus sequences are boxed.

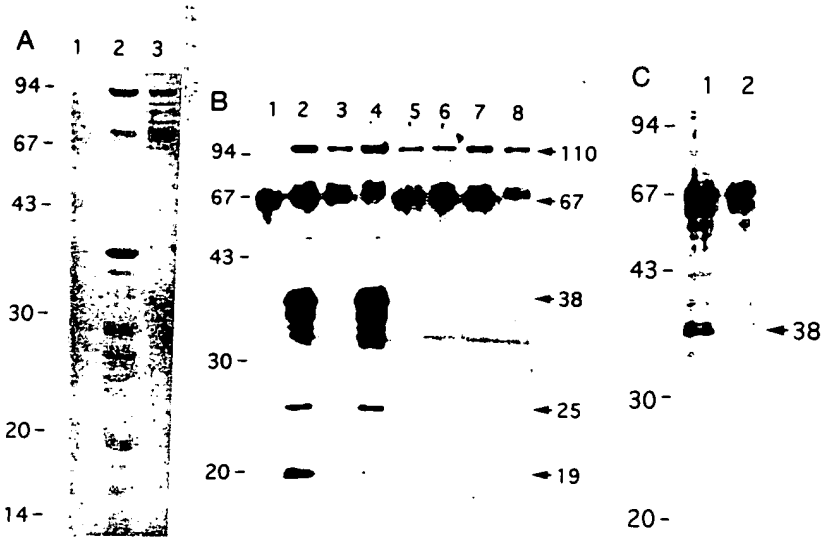

FIG. 3. (*A*) Coomassie blue-stained SDS/polyacrylamide gel of culture supernatants from *E. coli* HS-4 (lane 1), E2348/69 (lane 2), and CVD452 (lane 3). (*B*) Immunoblot of culture supernatants from *E. coli* HS-4 (lane 1), E2348/69 (lane 2), CVD452 (lane 3), CVD452(pCVD446) (lane 4), CVD452(pKJ2) (lane 5), CVD452(pKJ4) (lane 6), CVD452-(pKJ6) (lane 7), and CVD452(pBluescript) (lane 8), probed with rabbit antiserum against EPEC secreted proteins. (*C*) Whole cell extracts of E2348/69 (lane 1) and CVD452 (lane 2) probed with rabbit antiserum against EPEC secreted proteins. Molecular size (kDa) standards are indicated at left in each panel. Sizes (kDa) of bacterial secreted proteins are indicated at right.

110-kDa protein in supernatants, it does not recognize it in whole cell preparations, suggesting that this protein is secreted by a different mechanism than the other EPEC secreted proteins, a scenario consistent with the fact that mutation of *sepB* does not affect secretion of the 110-kDa protein.

FAS and LA. AE lesions can be visualized by the FAS test (1). The *sepA* mutation in 27-3-2(1) and the *sepB* mutation in CVD452 greatly reduced the ability of E2348/69 to cause AE lesions, with only a weak shadow of fluorescence evident (data not shown). When the *sepB* mutant was complemented with pCVD446, fluorescence was restored to levels similar to what was seen in HEp-2 cells infected with wild-type E2348/69 (Fig. 1). However, the smaller *sepB* subclones, pKJ2 and pKJ4, were not able to restore FAS activity to CVD452 (Fig. 1).

Wild-type E2348/69 exhibited a typical LA adherence pattern characterized by the formation of tight clusters of bacteria, referred to as microcolonies, on HEp-2 cells (32). The EPEC *sep* mutants CVD452 and 27-3-2(1) have altered LA patterns which appear as loose clusters of bacteria compared with the tight microcolonies formed by E2348/69 (data not shown). Plasmid pCVD446 restored a normal LA phenotype to CVD452, whereas plasmids pKJ2 and pKJ4 did not (Fig. 1).

Tyrosine Phosphorylation. EPEC induces tyrosine phosphorylation of three host cell cytoskeletal proteins, including a major band of 90 kDa (Hp90) and two minor bands of 72 kDa and 39 kDa (3). The *sepB* mutant CVD452 was unable to induce tyrosine phosphorylation of Hp90 or Hp72 (Fig. 4, lane 3). In our laboratory, the tyrosine-phosphorylated proteins induced by E2348/69 resolved as 88 kDa and 73 kDa (Fig. 4, lane 2) and neither band was present in control samples containing only bacteria (data not shown). pCVD446 restored the ability of CVD452 to induce tyrosine phosphorylation of Hp88 and Hp73 in HEp-2 cells (Fig. 4, lane 4). pKJ2 and pKJ4 failed to complement the *sepB* mutation for tyrosine phosphorylation (data not shown).

Immunoblots with Volunteer Serum. We tested the immunogenicity of the EPEC secreted proteins with serum drawn from a volunteer experimentally infected with E2348/69 (15). The postinfection serum, drawn 28 days after infection, strongly recognized the secreted proteins of E2348/69, whereas the preinfection serum did not (Fig. 5).

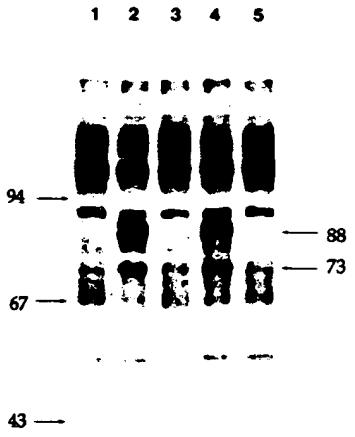

FIG. 4. Immunoblot showing 1% Triton X-100-soluble tyrosine-phosphorylated host cell proteins after 3.5 hr incubation of HEp-2 cells infected as follows: untreated (lane 1), E2348/69 (lane 2), CVD452 (lane 3), CVD452(pCVD446) (lane 4), CVD452(pBluescript) (lane 5). Molecular size (kDa) standards are indicated at left. The 88-kDa and 73-kDa proteins are indicated at right.

DISCUSSION

EPEC strains were the first *E. coli* to be associated with diarrheal disease. However, since the initial epidemiologic description of these organisms in 1945 (33), progress in elucidating mechanisms by which EPEC cause disease has lagged behind the discovery of pathogenic mechanisms of more recently described pathogens such as enterotoxigenic *E. coli* and enterohemorrhagic *E. coli* 0157:H7. The AE histopathology and cellular responses such as actin polymerization and protein phosphorylation have been described (1, 3), but it was not apparent how EPEC induced these changes in the eukaryotic cell. In this study, we report several genes, *sepA–sepD*, whose predicted protein products show striking similar-

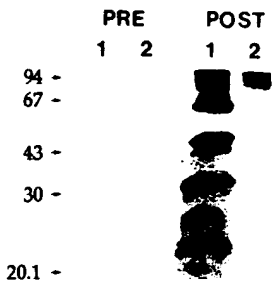

FIG. 5. Immunoblot showing the secreted proteins of E2348/69 (lane 1) and CVD452 (lane 2) recognized by human serum collected from a volunteer prior to infection with E2348/69 (PRE) and 28 days postinfection (POST). Molecular size (kDa) standards are indicated.

ities to components of a recently described type III secretory apparatus involved in extracellular protein secretion by many plant and animal pathogens such as *Yersinia*, *Shigella*, and *Salmonella* species (34–36). The proteins secreted by such systems, including the Yop (37) and Ipa (17, 38) proteins of *Yersinia* and *Shigella*, respectively, lack typical signal sequences and require the products of large gene clusters comprising at least 12 genes for their secretion.

The homology with type III secretory proteins led us to examine culture supernatants of EPEC strain E2348/69, where we found secreted proteins ranging in size from 110 kDa to 19 kDa. Secretion of all but the 110-kDa protein was abrogated by a mutation in the *sepB* gene, which encodes a predicted protein sequence similar to proteins that supply ATP in other type III secretion systems (22–25, 39). The secreted proteins, which are recognized by serum antibodies from an individual experimentally infected with E2348/69, are likely candidates for the diffusible EPEC product(s) previously hypothesized by Rosenshine *et al.* (3) to be involved in signal transduction leading to the AE histopathology. In an accompanying paper, Kenny and Finlay (40) independently found that E2348/69 secretes multiple polypeptides and determined that the amino-terminal sequence of a 38-kDa secreted protein matches the predicted sequence for the *eaeB* gene product (9), which is necessary for signal transduction (12). The predicted *eaeB* gene product lacks a typical signal sequence (9), which is consistent with it being secreted by a type III secretion pathway. Thus, there is strong evidence that the *sep* gene products are necessary for the secretion of essential virulence factors of EPEC. We presume that the altered phenotypes of 27-3-2(1) and CVD452 are due to the Tn*phoA* insertion in *sepA* and the *aphA3* insertion in *sepB*, respectively. However, since 27-3-2(1) has a second chromosomal Tn*phoA* insertion which could contribute to signal transduction defects, this conclusion should be qualified. It is somewhat puzzling that only one of our *sepB* subclones, pCVD446, was able to complement protein secretion, AE lesion formation, and tyrosine phosphorylation. Whether the failure to complement the mutation with the three smaller subclones pKJ2, pKJ4, and pKJ6 is due to a polar transcriptional effect or due to an imbalance in the normal ratio of the proteins of the type III secretion pathway is unknown.

In EPEC, the *sep* genes are located ≈12 kb from the gene, *eaeB*, encoding the secreted 38-kDa protein. These genes are located on a 35-kb region of DNA called LEE, which we have recently shown is highly conserved among bacterial pathogens producing the AE histopathology, including *E. coli* 0157:H7, the cause of recent outbreaks of hemorrhagic colitis and hemolytic uremic syndrome in the United States (7). The picture that is emerging for several bacterial pathogens is that there are blocks of plasmid or chromosomal DNA that encode both virulence determinants that interact with host cells and a specialized secretion apparatus for presenting these determinants to the cell. With EPEC and presumably other pathogens, thorough investigation of genetic sequences located nearby genes encoding such secretion apparatus should lead to the discovery of new virulence determinants which interact with host cells to cause disease.

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